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Respiratory and Nitrogenase Activities of Soybean Nodules Formed by Hydrogen Uptake Negative (Hup$^-$) Mutant and Revertant Strains of *Rhizobium japonicum* Characterized by Protein Patterns[1]

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ABSTRACT

Rates of respiratory CO_2 loss and nitrogenase activities of H_2 uptake-negative mutant strains and H_2 uptake-positive revertant strains of *Rhizobium japonicum* have been investigated. Two-dimensional gel protein patterns of bacteroids formed by inoculation of soybeans (*Glycine max* L.) with these two strains show that they are closely related and revealed only one obvious difference between them. On the basis of molecular weight standards, it was concluded that the missing protein spot in the H_2 uptake-negative mutant strain could be caused by a failure of the mutant to synthesize hydrogenase. Nodules formed by the H_2 uptake-negative mutant strain evolved respiratory CO_2 at a rate of about 10% higher than that of nodules formed by the H_2 uptake-positive revertant strain. During short-term experiments employed, rates of both C_2H_2 reduction and $^{15}N_2$ fixation varied considerably among replicate samples and no statistically significant differences between mutant and revertant strains were observed. It was observed that increasing the partial pressure of O_2 over nodules significantly decreased the proportion of nitrogenase electrons allocated to H^+.

Rhizobium bacteroids in root nodules of infected legumes convert atmospheric N_2 into ammonium that the plant uses for its growth. This reduction of N_2 requires energy supplied primarily in the form of carbohydrates from the photosynthesis of the host plant, of which the availability to the nodules is considered as a major factor limiting symbiotic N_2 fixation (16, 21). The nitrogenase, which catalyzes this reaction, reduces protons simultaneously (3, 19) and the portion of electrons diverted to proton reduction varies with the turnover rate of MoFe component of the enzyme (4). The nitrogenase reaction is the origin of the H_2 evolution by nodules first shown on soybeans in 1957 (18). According to surveys done so far, most of temperate *Rhizobium*-legume associations evolve significant amounts of this gas, with no known benefit (30–32, 33). H_2 evolution, therefore, is considered as a loss of energy, the extent of which averages about one-third of the electron flow through the nitrogenase (11).

Some associations do not evolve H_2 and the proportion of these appear higher among tropical legumes (32). In soybeans, 25% of the strains found in nodules of plants collected from various parts of the United States evolved little or no H_2 (23). This is due to a membrane-bound hydrogenase system carried by the bacteroids, which catalyzes the oxidation of H_2 to H_2O (5, 7, 28). According to its level of activity, the hydrogenase system recycles part or all of the H_2 formed by nitrogenase (8, 30). This oxyhydrogen reaction could benefit the N_2 fixation process by recovering part of the ATP and electrons expended in the conversion of protons to H_2, by functioning as a supplementary mechanism for the protection of nitrogenase from O_2 damage, and by preventing any inhibitory effect of H_2 in the immediate environment of nitrogenase (9).

The saving of carbohydrates in Hup$^+$[3] bacteroids supplied with H_2 (8, 26) and the recent discovery of H_2-supported chemolithotrophy of Hup$^+$ *R. japonicum* (15) demonstrate that Hup$^+$ strains of this species can use H_2 as a source of energy for their metabolic processes. Soybeans inoculated with a group of Hup$^+$ wild-type strains had 8.9% more crude protein in seed than the same cultivar associated with a group of wild-type Hup$^-$ strains in field experiments (13). The measure of the enhancement of symbiotic N_2 fixation from H_2 recycling at the plant level is now being investigated with Hup$^-$mutants and revertants obtained recently in this laboratory (22).

In this work, the protein patterns of two of these mutants and their revertants have been investigated by use of gel electrophoresis. The benefit of the H_2 recycling on the carbon economy of symbiotic N_2 fixation is then studied comparing respiration rates and nitrogenase activities of excised nodulated roots of a Hup$^-$ mutant and its Hup$^+$ revertant associated with a same soybean cultivar.

MATERIALS AND METHODS

Strains. The *Rhizobium japonicum* strains utilized were the Hup$^-$ mutants PJ17 and PJ18 isolated from a Hup$^+$ parental strain USDA 122 DES and their Hup$^+$ revertants PJ17-1 and PJ18-1, of which the reversion rates were consistent with those expected from point mutations (22).

Growth of Plants. Root nodules were obtained from plants grown either in Leonard jars with a one-fifth strength Jensen's nutrient solution (36) in a greenhouse (day/night temperature, 30°C–20°C; light intensity, 350 $\mu E \cdot m^{-2} \cdot h^{-1}$; 16-h photoperiod) or in 2 L of hydroponic solution (17) in a growth chamber (day/night temperature, 29°C–24°C; light intensity, 450 $\mu E \cdot m^{-2} \cdot h^{-1}$; 16-h photoperiod). In this case, 2 g of sterile $CaCO_3$ was added

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[3] Abbreviations: Hup$^+$, hydrogen-uptake positive; Hup$^-$, hydrogen-uptake negative; RE, relative efficiency; YEM, yeast extract mannitol.

per pot to control the pH, and pots were wrapped in aluminum foil to prevent any negative effect of light on nodulation and activity of the root system. The nutrient solution included 1 mM of urea during the first 28 d of growth. Seeds of soybean (*Glycine max.* L. cv Wilkin) were surface disinfected (36) and germinated on sterile agar plates containing mineral salts of a H_2 uptake medium. (25). Germinated seeds were inoculated after 24 or 48 h by soaking them for 1 h in inoculants of the above strains grown in yeast extract-mannitol liquid medium (36) and then transferred into Leonard jars or 2-L hydroponic containers, respectively.

Two d before experimental measurements, greenhouse plants were transferred to a growth chamber maintained under conditions described above.

Gel Electrophoresis. One-dimensional SDS gel electrophoresis was performed according to the Laemmli technique (20) with a 'mini slab' device (Idea Scientific Company, Corvallis, OR). Two-dimensional gel electrophoresis was conducted with a modification of O'Farrell technique (27) in which the first dimension was a non-equilibrium isoelectric focusing tube gel with reversed polarity and no pre-electrophoresis of ampholines.

Free-living *Rhizobium* were grown either in YEM or in H_2 uptake medium where they were derepressed for hydrogenase activity (25). Cell pellets were washed in 50 mM Tris-hydroxymethylaminomethanehydrochloride, 50 mM NaCl, 0.1% Nonidet P40 detergent (Calbiochem), pH 8.0, and then resuspended in an appropriate amount of isoelectric focusing buffer or SDS sample buffer of O'Farrell (27) for one- or two-dimensional gels. After bacteroid suspensions were prepared (7), they were purified by use of a sucrose gradient procedure (6) and then were broken by five successive cycles of freezing in liquid N_2 followed by thawing (34).

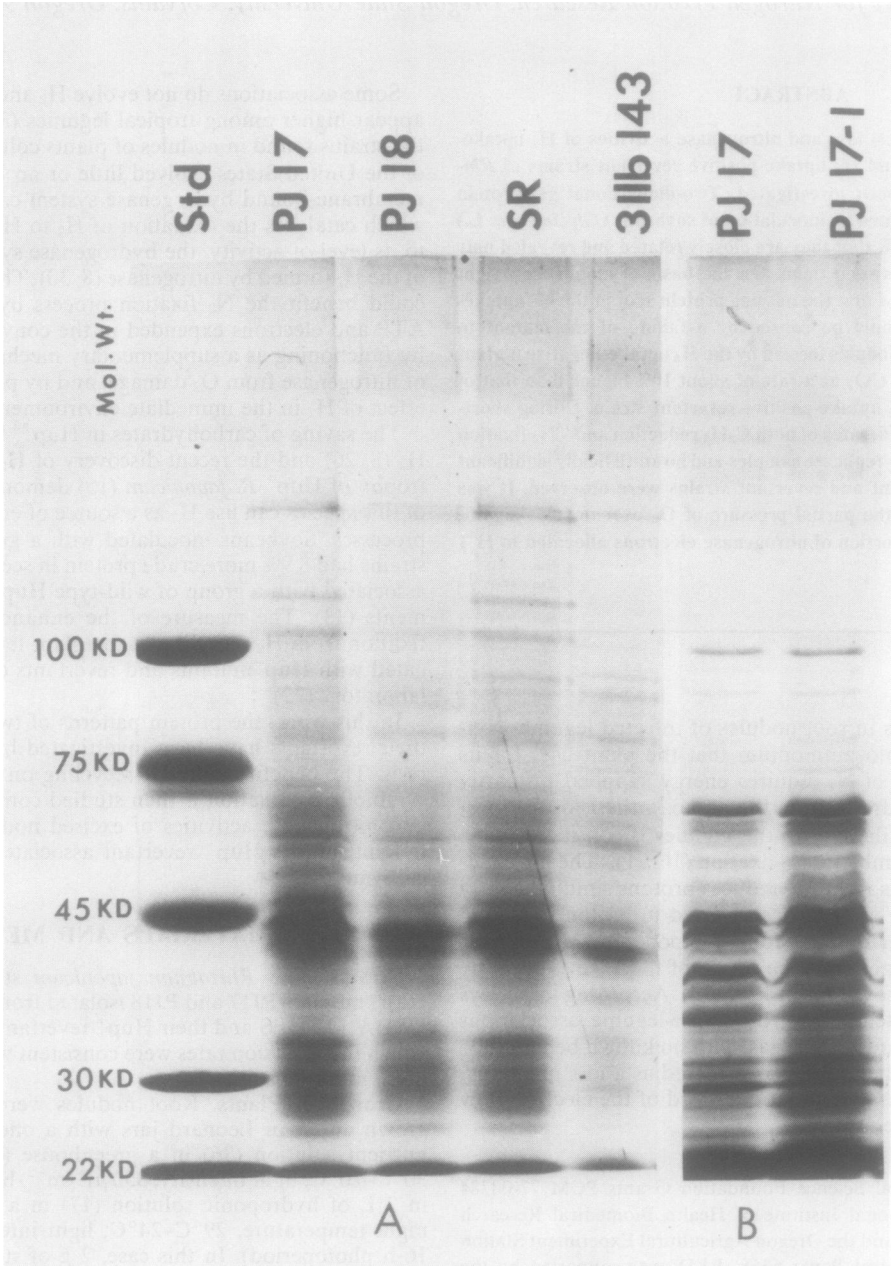

FIG. 1. One-dimensional SS acrylamide gradient gels (7.5–15%) of proteins in *R. japonicum* strains: A, Hup$^-$ mutant PJ17, Hup$^-$ mutant PJ18, Parent strain SR, Hup$^+$ wild-type strain 3I1b143 all grown to the exponential phase on YEM broth medium. B, Protein patterns of bacteroids from nodules formed by Hup$^-$ mutant strain PJ17 and revertant strain PJ17-1.

Table I. *Hydrogen Uptake by Derepressed Free-Living R. japonicum and Bacteroids of Hup⁻ Mutants PJ17 and PJ18 and Revertants PJ17-1, PJ18-1, and Their Parental Strain*

Protein content was determined by the Bio-Rad assay (Calbiochem). Results presented are means of two different free-living cultures derepressed for hydrogenase (26), and from a bacteroid suspension (7) prepared from 10 g of a fresh nodule sample obtained from five soybean plants.

Type of Cells Assayed	H_2 Uptake by *R. japonicum* Strains				
	SR	PJ17	PJ17-1	PJ18	PJ18-1
	nmol·mg⁻¹·protein h⁻¹				
Free-living	3.6	<0.03	6.7	<0.03	4.9
Bacteroids	0	<0.30	2,060.0	<0.30	2,592.0

Assays of H_2 Evolution, C_2H_2 Reduction, and CO_2 Evolution. Segments of the root crown (about 3.5 cm in length) with attached nodules were excised and 5 min later incubated as described previously (35) between 11 AM and 1 PM at 25°C. After 45 min of incubation, which was established as the limit of linear activities, nodules were excised and root respiration measured during a 15-min incubation. The rates of respiration of roots alone were in a range near 15 μmol CO_2 evolved $\cdot g^{-1}$ fresh root $\cdot h^{-1}$, corresponding to about 15% of nodule respiration in the experiments.

Initial and final composition of gases over the nodules were analyzed on a 5830A Hewlett-Packard chromatograph. For the measurement of C_2H_2 and C_2H_4, a 1.8 m × 3.2 mm column of Porapak R at a temperature of 42°C, a flow rate of N_2 carrier gas of 36 ml/min was used with a flame ionization detector. For H_2 determination, a 1.8 m × 6.4 mm column of molecular sieve 5 Å (40–60 mesh) at 120°C, a flow rate of N_2 carrier gas of 30 ml/min and a thermal-conductivity detector was used. H_2 uptake by free-living *Rhizobium* and bacteroids suspensions was measured with an amperometric method (14).

Respiration rates for short periods of incubation were obtained by determining CO_2 concentrations of the inner atmosphere over nodulated roots on a Carle 8500 chromatograph with a 52 cm × 3.2 mm column of Porapak Q at 72°C, a flow rate of helium carrier gas of 17 ml/min and a thermal conductivity detector. The volume of the incubation vessel was selected so that the final internal partial pressure of CO_2 never exceeded 1%. Greater concentrations of CO_2 are known to inhibit the nodule respiration (24). For longer incubations, a CO_2 trap was used consisting of a receptacle containing a folded filter paper to which was added by injection a 0.3 ml of 10 N NaOH solution at the beginning of the experiment. This maintained the inner partial pressure of CO_2 between 0.05 and 0.10%. At the end of each experiment, the trap was transferred into a serum bottle which was then sealed. The CO_2 was released by injecting 3 ml of 6 N H_2SO_4 into each bottle and agitation in a water bath at 20°C. After equilibration at 20°C, CO_2 concentration was determined by GC as described previously.

^{15}N Assay. Nodulated root segments were incubated for 45 min in an atmosphere containing $^{15}N_2$ using procedures described above and in the footnote of Table IV. At the end of the exposure, nodules were excised and stored at −70°C. After CO_2 evolution rates were determined on the roots, they also were stored at −70°C until they were analyzed. After drying samples at 80° for 24 h, total N was determined by the Kjeldahl method (2). The ^{15}N enrichment of the N in roots and nodules was determined by mass spectrometry at the Los Alamos National Laboratory.

RESULTS

Protein Patterns of Mutants and Revertants. Protein patterns on SDS slab gels of the Hup⁺ parent strain SR and its two mutants PJ17 and PJ18 were closely related compared to the 3Ib142 wild-type Hup⁺ strain of *R. japonicum* (Fig. 1A). Nevertheless, differences can be seen between PJ18 and SR which indicates that PJ18 is probably a multiple mutant of the parent strain SR, while PJ17 appears to be more closely related to the strain SR. In a second experiment in which gel electrophoresis was carried out on strains PJ17, PJ18 and their revertants from original stock cultures, sharp differences between mutants were observed again, but the protein patterns of each mutant and its revertant were apparently identical. This is consistent with the assumption that each mutant and revertant differ only in a point mutation affecting the hydrogenase presence and activity, and that the enzyme is not derepressed in cells cultured on the YEM medium.

Alternately, the failure to observe differences could be attributed to the limited resolving capacity of one-dimensional gels. Indeed, bacteroid one-dimensional gel protein patterns appear to be identical (Fig. 1B) even though the Hup⁺ revertant bacteroids had a very high hydrogenase activity, while the Hup⁻ bacteroid had no significant hydrogenase activity (Table I).

Two-dimensional protein patterns of PJ17 and PJ17-1 bacteroids appear to be very similar. Nevertheless, in replicate gels, only one spot observed in the PJ17-1 pattern was not observed in protein patterns of PJ17 (Fig. 2). On the basis of mol wt standards and the known mol wt of purified hydrogenase (1), this protein spot could be due to hydrogenase. The same protein spot was observed in the Hup⁺ revertant PJ18-1 and missing in the Hup⁻ mutant PJ18.

CO_2 Evolution and C_2H_2 Reduction of Root Nodules Formed by PJ17 and PJ17-1. In initial experiments, respiration and nitrogenase activities (C_2H_2 reduction) were measured on the same excised root nodule sample incubated during an initial 15 min in 40% O_2, 50% N_2, 10% Ar and then during a second 15-min period, in 40% O_2, 50% N_2, 10% C_2H_2. On 24, 30, and 38 d-old plants, C_2H_2 reduction rates of PJ17 and PJ17-1 nodules were not significantly different. At these dates, differences between the respiration rates of nodules formed by the two mutants also were not significant. The CO_2 evolved/C_2H_2 reduced was 4.54 ± 0.23 mol CO_2/mol C_2H_2 which did not vary appreciably during different vegetative stages of growth of the host plant from V_2 to V_6R_2 (12). At these stages, the yields of plants were not significantly different.

Additional measurements were conducted with plants less than 35 d old and respiratory CO_2 evolution was measured during an incubation period of 45 min by use of a CO_2 trap to prevent any inhibitory effect of CO_2 accumulation inside the incubation vessel (24). Respiration and acetylene reduction rates were measured on separate samples of nodulated roots because activities failed to remain linear for more than 50 min under the conditions used.

In experiments where nodules were supplied with 20% O_2, the nodules respiration of PJ17-1 was found to be less than that of PJ17 nodules. The difference of about 10%, however, was not significant at the 0.05 level. The experiments were then conducted under 40% O_2 where activities were considerably higher and presumably experimental errorless. Under these conditions, activities were increased by about 50% and the respiratory CO_2 evolution rate for PJ17 nodules was about 10% higher than rates for nodules formed by PJ17-1. Standard deviation, however, was 15% of the mean and the difference in CO_2 evolution rates was not statistically significant.

Nevertheless, after normalizing and combining data obtained with these two sets of experiments, the differences of 10.1% between the rates of CO_2 evolution by PJ17 and PJ17-1 nodules was significant in Student's t test at the 0.05 level (Table II). The acetylene reducing activity of PJ17-1 nodules was slightly lower than that of nodules formed by PJ17, but the difference was not significant. The rate of CO_2 evolution, divided by the rate of C_2H_2 reduction for PJ17-1 nodules, was 5.3% lower than the corresponding value for nodules formed by PJ17.

Fixation Rates of Nodules Formed by Hup⁺ and Hup⁻ Strains. In the above experiments, nitrogenase activities were measured by

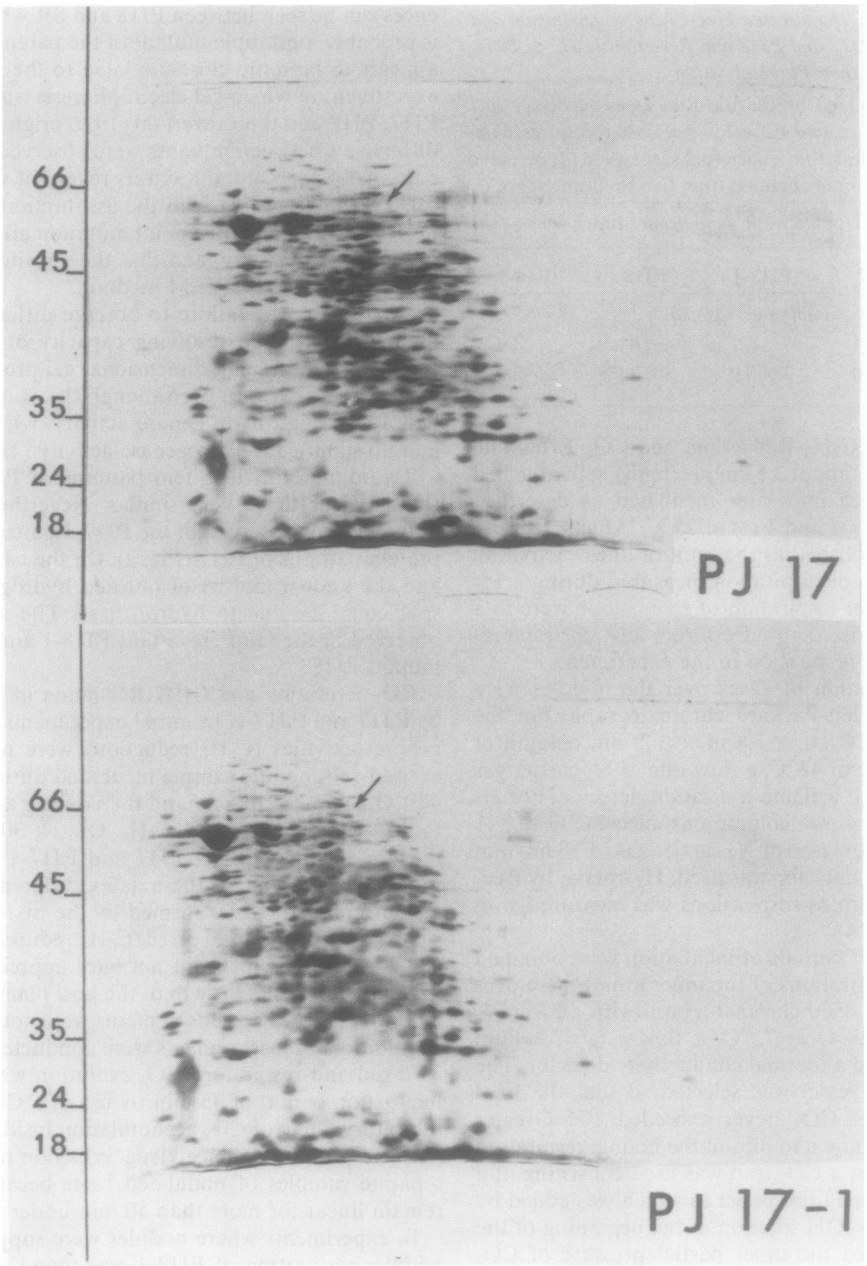

FIG. 2. Two-dimensional gel patterns of proteins in bacteroids of strains PJ17 and PJ17-1. The arrow shows a spot in the PJ17-1 protein pattern which is missing in the PJ17 protein pattern. The approximate mol wt of this protein is 64,000, a value which is approximately the same as that reported for purified bacteroid hydrogenase (1). The numbers at the left of the gel pattern are mol wt $\times 10^{-3}$ of standard proteins.

use of the C_2H_2 reduction assay. This method is widely used to estimate the total electron flow through the nitrogenase system, but acetylene at 0.1 atm is known to inhibit nitrogenase proton reduction. As a consequence, the acetylene reduction method is not considered as an accurate evaluation of the N_2 reducing activity, and it does not allow an evaluation of any direct effect of H_2 recycling on N_2 fixation *per se*.

An experiment was conducted, therefore, in which excised root nodules were incubated in an atmosphere containing $^{15}N_2$, while other samples were incubated at the same time in presence of 10% C_2H_2 (Table III). After 45 min of incubation, 20% of the $^{15}N_2$ fixed by the nodules formed by PJ17 and 16% of $^{15}N_2$ fixed by PJ17 nodules had been exported to the root tissues. In this experiment, nodules from plants inoculated with the Hup^- PJ17 fixed 33.9 ± 8 μg N whereas nodules formed by the Hup^+ PJ17-1 fixed 2918 ± 8.5 μg N in 45 min. This difference was not statistically significant.

The $^{15}N_2$ fixation rate of PJ17-1 nodules was lower than that of PJ17 nodules when expressed in $\mu mol \cdot g^{-1}$ fresh nodule $\cdot h^{-1}$, however, the difference was not significant (Table III). When fixation rates were expressed per mg of nodule protein, $^{15}N_2$ fixation rates were similar for the PJ17 mutant and its PJ17-1 revertant.

Assuming that the nitrogenase activity for samples of nodules incubated with $^{15}N_2$ was the same as that of nodules incubated with 10% C_2H_2, a ratio of C_2H_2 reduction rate/$^{15}N_2$ fixation (Table III) was 5.6 for PJ17 nodules and 5.9 for PJ17-1 nodules. These values are higher than the value of 3 expected from the theoretical consideration that the reduction of one molecule of N_2 requires three pairs of electrons while the reduction of C_2H_2 requires only one pair. In the absence of C_2H_2, however, part of the electron flow through nitrogenase was diverted toward proton reduction, while under 10% C_2H_2 almost all of the electrons were used for

Table II. *CO_2 Evolution and C_2H_2 Reduction of Soybean Nodules Formed by Hup⁻ Mutant (PJ17) and Hup⁺ Revertant (PJ1701) Strains of R. japonicum*

Nodulated roots were from plants 27 to 35 d old. CO_2 evolution and C_2H_2 reduction are expressed in $\mu mol \cdot g^{-1}$ fresh nodule $\cdot h^{-1}$. In the calculation of the ratio CO_2 evolved/C_2H_2 reduced, it was assumed that the C_2H_2 reducing activity was the same under argon or acetylene. Combined results are means with SE of 14 replicates of an experiment under 40% O_2, 40% N_2, 19.94% Ar, 0.06% CO_2 (absence of C_2H_2) or the same gas mixture when 10% C_2H_2 was added and/or Ar at 9.94% (presence of C_2H_2).

Observations	Nodules Formed by PJ17 Assayed in		Nodules Formed by PJ17-1 Assayed in	
	Absence of C_2H_2	Presence of 10% C_2H_2	Absence of C_2H_2	Presence of 10% C_2H_2
CO_2 evolution	61.46 ± 3.76	61.43 ± 2.75	55.22 ± 3.94	59.06 ± 3.59
C_2H_2 reduction		13.58 ± 1.30		12.88 ± 1.84
CO_2 evolved/C_2H_2 reduced	4.53	4.53 ± 0.11	4.29	4.59 ± 0.09

Table III. *$^{15}N_2$ Fixation, C_2H_2 Reduction, CO_2 Evolution, and H_2 Evolution of Soybean Nodules Formed by Hup⁻ Mutants (PJ17) and Hup⁺ Revertant (PJ17-1) Strains of R. japonicum under 40% O_2*

Excised nodulated roots were from plants 27 to 28 d old, assayed for 45 min under 40% O_2, 40% N_2, 9.94% AR, 10% C_2H_2 and 0.06% CO_2; or 40% O_2, 40% $^{15}N_2$, 19.94% Ar, and 0.06% CO_2. All results are expressed in $\mu mol \cdot g^{-1}$ fresh nodule $\cdot h^{-1}$ and are means and SE of five replicate samples of nodules.

Materials Assayed	$^{15}N_2$ Fixed	C_2H_2 Reduced	H_2 Evolved	CO_2 Evolved
Nodules formed by PJ17 assayed				
Presence of $^{15}N_2$	2.50 ± 0.30		5.15 ± 0.69	63.00 ± 2.19
Presence of C_2H_2		13.92 ± 0.76	0.59 ± 0.35	63.00 ± 2.69
Nodules formed by PJ17-1 assayed in				
Presence of $^{15}N_2$	2.23 ± 0.20		<0.10	56.96 ± 3.56
Presence of C_2H_2		13.06 ± 1.00	<0.10	58.84 ± 3.05

Table IV. *Effect of the Partial Pressure of O_2 on the Evolution, C_2H_2 Reduction, and RE of Soybean Nodules Formed by Hup⁻ Mutant (PJ17) and Hup⁺ Revertant (PJ17-1) Strains of R. japonicum*

Nodulated roots from 34-d-old plants were assayed under 40% N_2, 0.06% CO_2; Ar was used to adjust inner pressure to 1 atm in different treatments. RE was computed as: $RE = 1 - \frac{\text{rate of } H_2 \text{ evolution}}{\text{rate of } C_2H_2 \text{ reduction}}$. Results presented are means of four replicate samples of nodules.

Observations	Nodules Formed by PJ17 Assayed in				Nodules Formed by PJ17-1 Assayed in			
	20% O_2		40% O_2		20% O_2		40% O_2	
	No C_2H_2	10% C_2H_2	No C_2H_2	10% C_2H_2	No C_2H_2	10% C_2H_2	No C_2H_2	10% C_2H_2
H_2 evolution	4.21 ± 0.69	0.21 ± 0.20	5.59 ± 1.02	0.61 ± 0.44	<0.10	<0.10	<0.10	<0.10
C_2H_2 reduction		8.75 ± 0.78		14.49 ± 2.52		8.84 ± 0.73		13.24 ± 2.57
RE	0.52		0.65		>0.99		>0.99	

C_2H_2 reduction (Table III). Consequently, the rate of C_2H_2 reduction corresponding to the rate of N_2 fixation should be the total C_2H_2 reduction rate less the rate of H_2 evolved. A small correction is needed for the very low H_2 evolution rate under 10% C_2H_2, which was observed in this experiment. The ratio (C_2H_2 reduction rate − H_2 evolution rate in absence of C_2H_2 + the H_2 reduction rate in presence of C_2H_2) × ($^{15}N_2$ fixation rate)$^{-1}$ was 3.7 for PJ17 nodules.

Effect of O_2 Partial Pressure on RE. In all experiments, nodules formed by the Hup⁻ mutants strain PJ17 evolved H_2 at rapid rates. In the experiments carried out under 40% O_2, the RE value calculated according to Schubert and Evans (33) varied from 0.60 to 0.75. The higher values were obtained when nodules from younger plants were assayed, however, these differences were not statistically significant. On the other hand, RE values for PJ17 nodules exposed to 20% O_2 were consistently lower than those exposed to 40% O_2.

In order to examine the assumption that the pO_2 may affect RE value, PJ17 nodules from a group of plants grown in Leonard jars were incubated with a CO_2 trap for 45 min either in an atmosphere with 20% or 40% O_2 with and without 10% C_2H_2 (Table IV). The RE of nodules incubated under 40% O_2 was 0.66, a value that is significantly higher than the mean value 0.52 for the nodules tested under 20% O_2. When the pO_2 over nodules was increased from 20 to 40%, the H_2 evolution rate was increased about 25%, but the increase in rate of acetylene reduction was 75%. In contrast, nodules formed by the Hup⁺ strain PJ17-1 failed to evolve a measureable amount of H_2 in these experiments and the pO_2 had no effect on the hydrogenase activity. In atmospheres containing either 20 or 40% O_2, the hydrogenase system in nodules formed by strains PJ17-1 recycled all the H_2 evolved from the nitrogenase reaction.

DISCUSSION

We have investigated the nitrogenase activities, H_2 evolution and respiratory CO_2 evolution rates of nodules formed by Hup⁻

mutant and Hup$^+$ revertant strains of *R. japonicum*. In the presence of 10% C_2H_2 in the atmosphere over nodules, no significant differences in respiration rates between nodules formed by Hup$^-$ PJ17 and Hup$^+$ PJ17-1 strains were observed (Table II). These findings are not unexpected because the addition of C_2H_2 strongly inhibited H_2 evolution and, as a consequence, no H_2 was available for involvement in the H_2 recycling process. On the other hand, when nodules formed by strains PJ17 and PJ17-1 were incubated in an atmosphere lacking C_2H_2 (Ar substituted), the mean rate of CO_2 evolution from the nodules formed by the Hup$^-$ PJ17 was 10% higher than that of nodules formed by the Hup$^+$ PJ17-1 revertant strain. Assuming that the two strains differ only in their capacity to oxidize H_2, this observation shows a benefit of H_2 recycling which is in general agreement with the reports of Dixon (7) and Rainbird *et al.* (29). The differences that were observed, however, are not as great as those reported (29) for cowpea nodules formed by Hup$^+$ and Hup$^-$ wild-type strains. The 10% difference in respiratory CO_2 evolution rates of nodules formed by our PJ17-1 and PJ17 *R. japonicum* strains may be higher than expected from theoretical considerations. If one assumed that nodules formed by PJ17-1 recycled an amount of H_2 equivalent to the 5.15 μmol $H_2 \cdot g^{-1}$ fresh nodules$\cdot h^{-1}$, that 1 mol H_2 is oxidized by 0.5 mol O_2, and a respiratory quotient for soybean nodules of 0.82 (10), then this would be equivalent to a 3.14 μmol divided by a total respiratory CO_2 loss of 63 μmol$\cdot g^{-1}$ fresh nodules$\cdot h^{-1}$ amounts to a 5% theoretical expected difference between the Hup$^+$ and Hup$^-$ nodules. Of course, nothing is known about the possible regulatory role of H_2 oxidation on nodule respiratory metabolism or of other factors that are responsible for the differences in theoretical calculations and experimental observations.

Inasmuch as nodules formed by strain PJ17 showed no H_2 uptake activity, the total electron flow through nitrogenase can be measured by the C_2H_2 reduction rate and the quantity of electrons consumed in the reduction of N_2 can be measured by the difference: (C_2H_2 reduction rate $-$ H_2 evolution rate). The corresponding figure found here of 3.7 electron pairs needed to reduce one molecule of $^{15}N_2$ in PJ17 nodules is closer to the theoretical expectation of 3 than the value of 5.6 which was calculated without taking into consideration the H_2 evolution rate.

We have observed that PJ17 nodules, which recycle no H_2 have higher RE values under 40% O_2 than under 20% O_2. Apparently, this is associated with a decreased allocation of electrons to protons and an increased allocation of electrons to N_2 under the higher partial pressure of O_2. The turnover rate of the MoFe protein has been correlated with the allocation of electrons to various substrates. In experiments with purified nitrogenase components increasing, the ratio of Fe protein to MoFe protein or the concentration of Mg ATP resulted in a more rapid turnover rate of the MoFe protein and a decreased proportion of electrons transferred to protons (4). In our experiment, where the partial pressure of O_2 over PJ17 nodules was increased from 20 to 40%, there was an increased rate of C_2H_2 reduction and a lower rate of H_2 evolution showing an *in vivo* effect that could logically be explained on the basis of the work by Burris *et al.* (4) who showed a correlation between the turnover rate of purified MoFe protein and allocation of electrons to nitrogenase acceptors.

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